Klinik für Kleintiermedizin der Vetsuisse-Fakultät, Universität Zürich

Direktorin: Prof. Dr. Claudia Reusch, Dipl. ECVIM-CA

Arbeit unter Leitung von Dr. Peter Kook, Dipl. ACVIM & ECVIM-CA

Serum cobalamin, urinary methylmalonic acid and plasma homocysteine concentrations in healthy and cobalamin-deficient Border Collies

Inaugural – Dissertation

Zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Sabina Lutz

Tierärztin
von Wolfhalden AR, Schweiz

genehmigt auf Antrag von

Prof. Dr. Claudia Reusch, Dipl. ECVIM-CA, Referentin

Zürich 2011

Inhaltsverzeichnis

Seite

Zusammenfassung 3

Summary 4

Manuscript 5

- Abstract 6-7
- Introduction 8-9
- Materials and Methods 10-12
- Results 13-15
- Discussion 16-19
- Footnotes 20
- References 21-24
- Figures 1–3 25-27

Acknowledgements 28-29

Zusammenfassung

Beim Border Collie wird ein erblicher Cobalaminmangel vermutet. Die Diagnose beruht auf einer tiefen Cobalamin- und einer erhöhten Homozysteinkonzentration im Blut sowie auf einer erhöhten Methylmalonsäurekonzentration im Urin. Ziele dieser Studie waren (1) Referenzwerte für Cobalamin und Homozystein im Blut sowie für Methylmalonsäure im Urin (ausgedrückt als Quotient zum Kreatinin) zu erstellen und (2) Border Collies mit Hilfe dieser Parameter zu untersuchen.
Cobalamin wurde mittels Chemilumineszenz-Assay, Homozystein mittels HPLC mit fluorimetrischer Detektion und Methylmalonsäure mittels Gaschromatographie / Massenspektrometrie bestimmt. Insgesamt wurden 35 gesunde Hunde diverser Rassen und 113 Border Collies in die Studie aufgenommen. Vier Border Collies litten an einem Cobalaminmangel mit folgenden Wertebereichen: Cobalamin < 150 (Referenzbereich (Ref), 261.2–1001) ng/L, Homozystein 40–81.6 (Ref, 4.3–18.4) µmol/L und Methylmalonsäure 1800–6665 (Ref, < 4.2) mMol/Mol. Interessanterweise wiesen 37.7% der Border Collies mit normalem Cobalamin eine erhöhte Methylmalonsäurekonzentration auf ($P < 0.0001$). Zusammengefasst weist der Befund der Methylmalonazidurie bei Border Collies mit einer normalen Cobalaminkonzentration als auch bei solchen mit einem Cobalaminmangel auf 2 verschiedene biochemische Defekte hin. Studien, die die Cobalaminabsorption und dessen Stoffwechselwege untersuchen, sind indiziert.

Keywords: Cobalaminmangel, Methylmalonazidurie, Border Collie

Summary

Hereditary cobalamin deficiency is suspected in the Border Collie breed. Diagnosis is based on hypocobalaminemia, hyperhomocysteinemia and methylmalonic aciduria. Goals of the study were (1) to establish reference values for the blood concentrations of cobalamin and homocysteine and for the concentration of urinary methylmalonic acid and (2) to screen a larger Border Collie population with the aforementioned markers.

Cobalamin, homocysteine and methylmalonic acid were measured using an automated chemiluminescence assay, HPLC with fluorimetric detection and gas chromatography / mass spectrometry. A total of 113 Border Collies and 35 healthy dogs of different breeds were examined. Four Border Collies suffered from cobalamin deficiency with the following concentrations: cobalamin < 150 (reference range (ref), 261–1001) ng/L, homocysteine 40–81.6 (ref, 4.3–18.4) µmol/L, and methylmalonic acid 1800–6665 (ref, < 4.2) mmol/mol. Unexpectedly 37.7% of Border Collies with normal cobalamin had significantly higher methylmalonic acid concentrations ($P < 0.0001$). In conclusion, the simultaneous finding of methylmalonic aciduria in Border Collies with normal cobalamin concentrations in addition to Border Collies with clinicopathologic findings of cobalamin deficiency is surprising and suggests two different defects. Future studies investigating the absorption process as well as the metabolic pathway of cobalamin are warranted.

Keywords: Cobalamin deficiency, Methylmalonic aciduria, Border Collie

Serum cobalamin, urinary methylmalonic acid and plasma homocysteine concentrations in healthy and cobalamin-deficient Border Collies

Sabina Lutz, med vet; Adrian C. Sewell, Dr; Beat Bigler, Dr med vet; Barbara Riond, Dr med vet; Claudia E. Reusch, Prof Dr med vet; Peter H. Kook, Dr med vet

From the Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, Switzerland (Lutz, Reusch, Kook); the Department of Pediatrics, University of Frankfurt, Germany (Sewell); the Laupeneck Laboratory, Bern, Switzerland (Bigler); the Institute for Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Switzerland (Riond).

Supported by a grant from the Albert-Heim-Foundation.

Presented as an oral presentation at the 29th Annual ACVIM Forum Denver, CO, 2011.

The authors thank Prof. Ralph Gräsbeck for helpful discussions.

Address correspondence to Dr. Kook (peterhendrikkook@gmail.com)

Abstract

Objective–To establish reference values for serum cobalamin (Cbl), urinary methylmalonic acid/creatinine ratios (uMMA/Cr) and plasma total homocysteine (tHcy) in healthy pet dogs and to evaluate these biomarkers in the Border Collie (BC), a breed in which hereditary cobalamin deficiency (CD) has been described.

Animals–One hundred thirteen BC and 35 control dogs.

Procedures–Prospective study. Serum Cbl, urinary MMA and plasma tHcy were measured using an automated chemiluminescence assay, gas chromatography/mass spectrometry, and HPLC with fluorimetric detection, respectively.

Results–Four BC with Cbl concentrations below the detection limit of 150 ng/L (reference range, 261–1001) were identified. In these 4 BC the median uMMA/Cr was 4064 mmol/mol (reference range, < 4.2), and the median tHcy was 51.5 µmol/L (reference range, 4.3–18.4). Clinicopathologic signs included stunted growth, lethargy, anemia, and proteinuria. All dogs improved markedly with regular Cbl supplementation. Of the 109 healthy BC with normal Cbl and tHcy values, 41 (37.7%) had significantly ($P < 0.0001$) higher uMMA/Cr compared to control dogs ranging from 5 to 360 mmol/mol.

Conclusions and Clinical Relevance–Hereditary CD is a rare disease with variable clinical signs in the BC. The concurrent finding of methylmalonic aciduria in healthy eucobalaminemic BC in addition to sick BC diagnosed with CD is surprising and

suggests two different defects: intestinal Cbl malabsorption or defects in the intracellular processing of Cbl. Future studies investigating the absorption process as well as the metabolic pathway of Cbl are warranted.

Abbreviations

BC	Border Collie
CBC	Complete blood cell count
Cbl	Cobalamin
CD	Cobalamin deficiency
CV	Coefficient of variation
uMMA/Cr	Urinary methylmalonic acid/creatinine ratio
tHcy	Total homocysteine

Introduction

Cobalamin (Cbl) (vitamin B12) is an essential cofactor for several enzyme systems in mammalian species, and adequate amounts are required for nucleic acid synthesis.[1] Animals are unable to synthesize Cbl and therefore entirely dependent upon adequate

dietary sources.[1] The absorption of Cbl is complex, as it is first bound to haptocorrin, then to gastric or pancreatic intrinsic factor, and finally transferred to specific receptors located on the ileal enterocytes.[2] Hypocobalaminemia can develop for several reasons, including pancreatic and intestinal disease.[3] In humans, cobalamin deficiency (CD) due to selective malabsorption is a rare autosomal-recessive hereditary disorder appearing initially in early childhood.[4,5] In dogs, hereditary CD has been reported in Giant Schnauzers, Australian Shepherds, and in Chinese Shar Peis.[6-8] Moreover, CD has been repeatedly described in the Border Collie (BC) breed,[9-11] as well as in one Beagle.[12] Cobalamin acts as a co-factor in the conversion of methylmalonyl-CoA to succinyl-CoA via the enzyme methylmalonyl-CoA mutase and is needed for the re-methylation of homocysteine via the enzyme methionine synthase.[1] Deficiency of Cbl leads to reduced activity of both of these enzymes resulting in an increase of methylmalonic acid (MMA) and total homocysteine (tHcy).[1] Measurement of these metabolites allows the assessment of cellular Cbl availability and is the test of choice to detect early or mild CD in humans.[13] Correlations of urinary methylmalonic acid/creatinine (MMA/Cr) ratios and plasma tHcy with serum Cbl levels have not been investigated in dogs so far. Also, existing reference ranges for Cbl have not been compared with concurrent measurements of these cellular markers. After having diagnosed CD in BC presenting with nonspecific clinical signs,

the authors hypothesized that this deficiency might be more prevalent than actually recognized.

Thus the goals of this study were (1) to establish reference values for serum Cbl, urinary uMMA/Cr and tHcy in healthy pet dogs and (2) to evaluate these markers of Cbl metabolism in the BC breed.

Materials and Methods

This study was approved by the Committee for the Permission of Animal Experimentation, Canton of Zurich, Zurich, Switzerland.

BC–Between July 2009 and September 2010, 113 purebred BC were screened for CD. Dog owners were recruited for participation through the Swiss BC homepage, articles in Swiss dog magazines and through informed referring veterinarians. Assessment of all dogs included a detailed history, physical examination, complete blood cell count (CBC), serum biochemistry and urinalysis.

Control dogs from other breeds–Thirty-five healthy dogs were recruited as controls. Inclusion criteria were (1) being a breed other than a BC or BC cross (2) no history of disease in the past 12 months and judged to be healthy by their owners (3) normal physical examination (4) unremarkable CBC, serum biochemistry, and complete urinalysis. The group consisted of 19 mixed-breed dogs, 3 Labrador Retrievers, 2 Golden Retrievers, and 11 other pedigree breeds. The median age was 5 years (range, 1–15), and the median bodyweight was 12.6 kg (range, 5.1–43). There were 9 female, 5 male, 9 spayed female and 12 neutered male dogs.
All dogs were fasted 8 to 12 hours before blood sampling. Urine samples were collected by the owner in the evening or morning before the examination. A paired urine sample (fasted and 8 h post standard meal) for assessment of the effect of prior food intake on urinary MMA excretion was analyzed in 6 dogs.
Serum Cbl, urine uMMA/Cr und plasma tHcy concentrations were additionally determined in 12 supplementary healthy dogs that were exclusively fed bone and raw food. Breeds included 2 Australian Shepherds, 1 Jack Russell Terrier, 1 Alaskan

Malamute, 1 Tervueren, 1 Airedaile Terrier and 6 mixed-breed dogs. The median age was 5.4 years (range 1.9–13.3) and the median bodyweight was 22.7 kg (range, 6.1–39.7). There were 1 female, 1 male, 7 spayed female and 3 neutered male dogs.

Serum Cbl, plasma tHcy, and uMMA/Cr–Serum Cbl was measured using an automated chemiluminescence assay[a] as described before.[8] The upper limit of detection of this assay is 1,000 ng/L, and serum samples were diluted 1:2 or higher if necessary. The in-house intra- and interassay coefficients of variation (CV) for canine serum samples were 2.1% and 3.4%, respectively. The lower detection limit of the assay is 150 ng/L.

Plasma tHcy was measured using high performance liquid chromatography (HPLC) and fluorimetric detection.[14] Blood samples, collected in pre-chilled sodium citrate tubes, were immediately centrifuged at 1570 g at 4°C for 10 min. The plasma was separated and stored at -80°C until assayed. Homocysteine was added to a canine citrate plasma pool to give a concentration of 100 µmol/L. This pool sample was sequentially diluted to give standards of 50, 25, 12.5, 5.0 and 2.5 µmol/L (aliquots were stored at -80°C) and a standard curve was run with each batch of samples. Recoveries were tested by including 3 standards (25, 12.5 and 5.0 µmol/L) as samples five times during a 3-week period. The recoveries were > 96% for each standard tested. As no quality control material for tHcy is commercially available in canine samples, we included a canine plasma pool in each run (mean concentration = 16.8 µmol/L). The between run CV for this sample was < 6%. The within batch CV was < 3% at a concentration of 50 µmol/L and < 6% at 5 µmol/L. The lower limit of detection was 2.5 µmol/L.

Urinary MMA was determined by gas chromatography/mass spectrometry[b] with a lower limit of detection of 0.15 mmol.[15] Results were expressed per mol of urinary creatinine. Creatinine was measured by the Jaffe method using an ABXPentra 400 analyzer.[c] This method had been validated for canine samples using the same instrument at the University School of Veterinary Medicine, Giessen, Germany.

Statistical analysis–Data were analyzed using GraphPad PRISM 5.0.[d] Each data set was evaluated for normality by Kolmogorov-Smirnov test. Within the two groups Cbl, uMMA/Cr, tHcy, results of CBC and serum biochemistry were compared using the Mann-Whitney U-test. The Spearman's rank correlation coefficient was used to determine a relationship between uMMA/Cr, Cbl and tHcy in both groups. Values of $P < 0.05$ were considered statistically significant. Reference ranges were established using the nonparametric percentile method. The 2.5 and 97.5 percentiles were determined to achieve the 95% double-sided reference interval in case of Cbl and tHcy. Regarding uMMA/Cr, the 95th percentile was used to obtain the one-sided reference range. Serum Cbl concentrations and uMMA/Cr outside the working range of the assay were assumed to be 149 ng/L and 1.9 mmol/mol, respectively.

Results

Control dogs–Serum Cbl concentrations ranged from 261–1001 ng/L (median, 441 [mean ± SD; 540.5 ± 235.5] Figure 1). The established reference range was 261–1001 ng/L, calculated from the central 95th percentile.

Urinary MMA/Cr ranged from < 2–6.6 mmol/mol (median, 1.9 [mean ± SD; 2.1 ± 0.8] Figure 2); 32 dogs had uMMA/Cr < 2 mmol/mol, 2 dogs had 2.5 and 3.6 mmol/mol respectively. The established upper reference limit was 4.2 mmol/mol. Previous food intake had no effect on uMMA/Cr in 6 dogs; all paired samples were < 2 mmol/mol.

Plasma tHcy concentrations ranged from 4.3–18.4 µmol/L (median, 9.1 [mean ± SD; 10.4 ± 4.5] Figure 3). The calculated reference range (central 95th percentile) was 4.3–18.4 µmol/L.

No correlation was detected with the Spearman's rank correlation coefficient between Cbl and tHcy as well as between Cbl and uMMA/Cr and uMMA/Cr and tHcy.

Results of dogs that were exclusively fed bone and raw food did not differ from results of control dogs.

Border Collies

Healthy BC–Data of 109 healthy BC were analyzed. None of the dogs received any supplements at the time of the study. All dogs were physically in athletic shape and no abnormalities were noted upon clinical examination. Hematologic, biochemical, and urine examinations were unremarkable in all 109 dogs. The median age was 4 years (range, 0.2–14) and the total group consisted of 32 intact male, 30 intact female, 28 spayed female and 19 neutered male dogs. The median body weight was 17.3 kg (range, 2.7–29). The median serum Cbl concentration was 592 ng/L (range,

150–1855 [mean ± SD; 641.4 ± 304.5] Figure 1), which was not significantly different compared to control dogs.

Urinary MMA/Cr ranged from < 2–360 mmol/mol (median, 1.9 [mean ± SD; 23.7 ± 60.1] Figure 2), 47 (43.1%) BC had results > 2 mmol/mol (range, 3.2–360 mmol/mol) and 41 (37.7%) had uMMA/Cr above the upper reference limit of 4.2 mmol/mol. The uMMA/Cr were significantly higher ($P < 0.0001$) compared to controls.

The urinary creatinine concentrations of 41 BC with elevated uMMA/Cr were not significantly different compared to 68 BC with uMMA/Cr within the reference range.

Plasma tHcy values ranged from 2.8–22.4 µmol/L (median, 8.5 [mean ± SD; 9.5 ± 4] Figure 3) and were not different from those of control dogs.

Five healthy BC had Cbl values below the reference range (261–1001 ng/L) ranging from 150–259 ng/L (median, 251). All of these 5 BC had uMMA/Cr and tHcy values within the reference range.

Cbl and tHcys concentrations of the 47 healthy BC with uMMA/Cr > 2 mmol/mol did not differ significantly compared to controls. The Spearman's rank correlation coefficient did not reveal any correlation between the aforementioned three parameters (Cbl, tHcys, uMMA/Cr) in all healthy BC as well as in BC with uMMA/Cr above the upper reference limit.

BC with CD–CD was diagnosed in 4/113 BC. The median age was 11.5 months (8–42), the median weight was 11.6 kg (11–12.1) and all dogs were intact females. All dogs had serum Cbl concentrations < 150 ng/L (Figure 1), the median uMMA/Cr was 4064 mmol/mol (range, 1800–6665; Figure 2), and the median plasma tHcy concentration was 51.5 µmol/L (range, 40–81.6; Figure 3). All 4 dogs were fed different commercial dog foods.

Affected BC exhibited growth failure (4/4), lethargy (4/4), glossitis (2/4), febrile episodes (1/4), mild non-regenerative anemia (3/4), neutropenia (1/4), isolated elevated aspartate aminotransferase activities (3/4) and mild proteinuria (4/4). Parenteral cobalamin administration produced complete remission of all clinicopathologic abnormalities, even though proteinuria and isolated aspartate-aminotransferase activity elevations remained.

Discussion

To the authors knowledge, serum Cbl concentrations in direct comparison with its cellular biomarkers MMA and tHcy have not been evaluated in healthy pet dogs so far. Details of currently used reference ranges have not been published. Results of the additional measurements of these Cbl biomarkers confirm the hitherto existing serum Cbl reference range. Although no biochemical gold standard exists to predict Cbl status, a normal MMA value in humans is generally considered supportive of a normal Cbl status, even when Cbl concentration is low.[16] Little is known about MMA in dogs. Elevated serum MMA concentrations predicted serum Cbl status in cats and decreased again with Cbl supplementation.[17] Similarly, Berghoff et al. recently documented a negative correlation between serum Cbl and serum MMA concentrations in dogs.[18] Results of that study also suggested that measurement of serum MMA concentration may be a better diagnostic test for CD than serum Cbl concentration. Urinary MMA has only sporadically been measured, and no reference ranges have been established so far.[9,10,12,19] Measurement of uMMA/Cr may have several advantages. Firstly, MMA values in urine are up to 40 fold higher than in serum and therefore easy to detect.[20] Secondly, urinary MMA is expressed as a ratio to urinary creatinine, thereby minimizing influences from hemoconcentration and kidney disease.[20,21] Thirdly, MMA is very stable in urine,[22] whereas no data exist on serum MMA stability. Lastly, a free catch urine sample might be less invasive and easily obtainable by owners compared to blood sampling.

Unexpectedly, uMMA/Cr in healthy BC were significantly higher compared to controls. Causes for elevated uMMA/Cr in people include prior food intake, although postprandial levels have only been shown to rise as high as 3 mmol/mol.[23] A diet-induced effect seems unlikely in our study as sampling conditions were identical for

both groups. Furthermore, uMMA/Cr investigated separately in 6 staff-owned dogs before and after feeding a standard meal did not differ. Even if diet had a minor impact on elevated uMMA/Cr of healthy BC, our observed values are still much higher than those reported in non-fasted humans.[23]

Theoretically, small intestinal bacterial overgrowth may also increase urinary MMA excretion. An overgrowth of bacteria producing propionic acid, a precursor of methylmalonyl-CoA, could lead to increased formation of urinary MMA.[24] The authors cannot fully exclude this possibility, but deem this rather unlikely, as none of the healthy BC had a history of digestive problems. Most notably, feeding patterns did not differ between control dogs and BC.

Extremely high uMMA/Cr (237, 264, and 360 mmol/mol) were found in 3 healthy un-related BC living in the same household. All dogs were fed with bone and raw food. Because feeding bone and raw food usually comprises a freeze-thaw process, loss of water-soluble B vitamins was suspected. In order to clarify this, serum Cbl, and plasma tHcy concentrations as well as uMMA/Cr of 12 additional healthy pet dogs exclusively fed bone and raw food were determined. Results did not differ compared to control dogs.

It is possible that the healthy eucobalaminemic BC with methylmalonic aciduria represent subclinical carriers of hereditary selective Cbl malabsorption. Genetic testing would be required to verify this hypothesis. However lacking differences in serum Cbl and plasma tHcy concentrations between control dogs and healthy BC make a carrier status appear less likely.

In humans, inborn errors of cellular Cbl metabolism are further reasons for methylmalonic aciduria.[25,26] Intracellular Cbl metabolism involves multiple steps between the lysosomal release of Cbl and the synthesis of adenosylcobalamin in the

mitochondria (required by the mitochondrial enzyme methylmalonyl-CoA mutase) and methylcobalamin in the cytosol (required by the cytoplasmic enzyme methionine synthase). To date, nine distinct defects of this pathway have been defined in humans leading either to isolated methylmalonic aciduria or to isolated homocysteinemia or both, depending on which step in metabolism is affected.[25,26] In these individuals, Cbl levels are usually in the reference range, as observed in our healthy BC group. However, in people the majority of defects are usually associated with overt clinical signs, leading to life-threatening disease, whereas asymptomatic affected individuals with methylmalonic aciduria are very rare.[26]

In this regard, our observation of increased uMMA/Cr in 37.7% of all screened BC could represent a rare phenomenon called benign methylmalonic aciduria. Benign methylmalonic aciduria has been reported in children without evidence of CD and without response to the administration of Cbl.[27] Two siblings in that study, were found to have a defect in the methylmalonyl-CoA mutase enzyme.[27] Another report described benign methylmalonic aciduria in a Turkish family, where three family members had normal serum Cbl concentrations, normal plasma and urine tHcys concentrations. Results of an extended biochemical screening for other known causes of methylmalonic aciduria were all normal, including an intact methylmalonyl-CoA mutase system.[28]

All BC with CD had elevated plasma tHcy concentrations compared to controls. Homocysteine is the intermediate product of methionine metabolism; its further metabolism is Cbl-dependent. Homocysteine is a very sensitive indicator of CD in humans and levels rise early in the course of disease often preceding clinical signs. Renal disease, hemoconcentration, thyroid disease, folate deficiency and drugs are known causes for hyperhomocysteinemia.[29] Similarly increased tHcy levels were

associated with renal and cardiac diseases in one study in dogs.[30] None of these potential causes were found in the BC with CD.

Interestingly none of the 41 healthy BC dogs with elevated uMMA/Cr had elevated tHcy values, thus making a subclinical defect in the methylmalonic-CoA mutase more likely.

Hypocobalaminemia (range, 150–259 ng/L; median, 251 [reference range 261-1001]) was also documented in 5 healthy BC with 4 dogs having nearly normal Cbl values (230, 251, 254, and 259 ng/L). Unlike the 4 diseased BC with CD, these hypocobalaminemic healthy BC had normal uMMA/Cr and plasma tHcy values. Also in sharp contrast to the diseased BC, these dogs were in excellent physical and clinical condition. The possibility of enzyme-bound tissue Cbl preventing cellular deficiency further indicates the necessity to measure cellular Cbl markers.[12]

In conclusion, the concurrent finding of isolated methylmalonic aciduria in healthy BC with normal Cbl concentrations and sick BC suffering from CD is intriguing and awaits further clarification. These results may suggest different disease processes: A defect in the mitochondrial metabolic pathway of Cbl (i.e. methylmalonyl-CoA mutase) on the one hand, and a selective intestinal malabsorption of Cbl on the other hand. Future studies should focus on genetic testing, intestinal Cbl absorption, as well as on methylmalonyl-CoA mutase functions.

Footnotes

a. Immulite 2000, Vitamin B12, Siemens Healthcare Diagnostics Inc.

b. Shimadzu QP5050A.

c. AxonLab, Stuttgart, Germany.

d. GraphPad Prism 5.0, GraphPad, San Diego, CA.

References

1. Markle HV. Cobalamin. *Crit Rev Clin Lab Sci* 1996;33:247-356.

2. Marcoullis G, Rothenberg SP. Intrinsic factor-mediated intestinal absorption of cobalamin in the dog. *Am J Physiol* 1981;241:294-299.

3. Batt RM, Morgan JO. Role of serum folate and vitamin B12 concentrations in the differentiation of small intestinal abnormalities in the dog. *Res Vet Sci* 1982;32:17-22.

4. Grasbeck R, Gordin R, Kantero I, et al. Selective vitamin B12 malabsorption and proteinuria in young people. A syndrome. *Acta Med Scand* 1960;167:289-296.

5. Imerslund O. Idiopathic chronic megaloblastic anemia in children. *Acta Paediatr Suppl* 1960;49:1-115.

6. Fyfe JC, Hall CA, Jezyk PF, et al. Inherited selective intestinal cobalamin malabsorption and cobalamin deficiency in dogs. *Pediatr Res* 1991;29:24-31.

7. He Q, Madsen M, Kilkenney A, et al. Amnionless function is required for cubilin brush-border expression and intrinsic factor-cobalamin (vitamin B12) absorption in vivo. *Blood* 2005;106:1447-1453.

8. Grützner N, Bishop MA, Suchodolski JS, et al. Association study of cobalamin deficiency in the Chinese Shar Pei. *J Hered* 2010;101:211-217.

9. Morgan LW, McConnell J. Cobalamin deficiency associated with erythroblastic anemia and methylmalonic aciduria in a border collie. *J Am Anim Hosp Assoc* 1999;35:392-395.

10. Battersby IA, Giger U, Hall EJ. Hyperammonaemic encephalopathy secondary to selective cobalamin deficiency in a juvenile Border collie. *J Small Anim Pract* 2005;46:339-344.

11. Outerbridge CA, Myers SL, Giger U. Hereditary Cobalamin Deficiency in Border Collie Dogs. *J Vet Intern Med* 1996;10:169.

12. Fordyce HH, Callan MB, Giger U. Persistent cobalamin deficiency causing failure to thrive in a juvenile beagle. *J Small Anim Pract* 2000;41:407-410.

13. Savage DG, Lindenbaum J, Stabler SP, et al. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. *Am J Med* 1994;96:239-246.

14. Vester B, Rasmussen K. High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. *Eur J Clin Chem Clin Biochem* 1991;29:549-554.

15. Sewell AC, Böhles HJ. 4-Hydroxycyclohexanecarboxylic acid: a rare compound in urinary organic acid analysis. *Clin Chem* 1991;37:1301-1302.

16. Vogiatzoglou A, Oulhaj A, Smith AD, et al. Determinants of plasma methylmalonic acid in a large population: implications for assessment of vitamin B12 status. *Clin Chem* 2009;55:2198-2206.

17. Ruaux CG, Steiner JM, Williams DA. Early biochemical and clinical responses to Cbl supplementation in cats with signs of gastrointestinal disease and severe hypocobalaminemia. *J Vet Intern Med* 2005;19:155-160.

18. Berghoff N, Suchodolski JS, Steiner JM. Association of serum Cbl and methylmalonic acid concentrations in dogs. *Vet J* 2011 Apr 6. [Epub ahead of print]

19. Fyfe JC, Jezyk PF, Giger U, et al. Inherited selective malabsorption of vitamin B12 in Giant Schnauzers. *J Am Anim Hosp Assoc* 1989;25:533-539.

20. Norman EJ, Cronin C. Cobalamin deficiency. *Neurology* 1996;47:310-311.

21. Norman EJ. Urinary methylmalonic acid/creatinine ratio: a gold standard test for tissue vitamin B12 deficiency. *J Am Geriatr Soc* 1999;47:1158-1159.

22. Matchar DB, Feussner JR, Millington DS, et al. Isotope-dilution assay for urinary methylmalonic acid in the diagnosis of vitamin B12 deficiency. A prospective clinical evaluation. *Ann Intern Med* 1987;106:707-710.

23. Rasmussen K. Studies on methylmalonic acid in humans. I. Concentrations in serum and urinary excretion in normal subjects after feeding and during fasting, and after loading with protein, fat, sugar, isoleucine, and valine. *Clin Chem* 1989;35:2271-2276.

24. Bain MD, Jones M, Borriello SP, et al. Contribution of gut bacterial metabolism to human metabolic disease. *Lancet* 1988;1:1078-1079.

25. Whitehead VM. Acquired and inherited disorders of cobalamin and folate in children. *Br J Haematol* 2006;134:125-136.

26. Watkins D, Rosenblatt DS. Inborn errors of cobalamin absorption and metabolism. *Am J Med Genet C Semin Med Genet* 2011;157:33-44.

27. Ledley FD, Levy HL, Shih VE, et al. Benign methylmalonic aciduria. *N Engl J Med* 1984;311:1015-1018.

28. Sewell AC, Herwig J, Böhles H. A case of familial benign methylmalonic aciduria? *J Inherit Metab Dis* 1996;19:696-697.

29. Stanger O, Herrmann W, Pietrzik K, et al. DACH-LIGA homocystein (german, austrian and swiss homocysteine society): consensus paper on the rational clinical use of homocysteine, folic acid and B-vitamins in cardiovascular and thrombotic diseases: guidelines and recommendations. *Clin Chem Lab Med* 2003;41:1392-1403.

30. Rossi S, Rossi G, Giordano A, et al. Homocysteine measurement by an enzymatic method and potential role of homocysteine as a biomarker in dogs. *J Vet Diagn Invest* 2008;20:644-649.

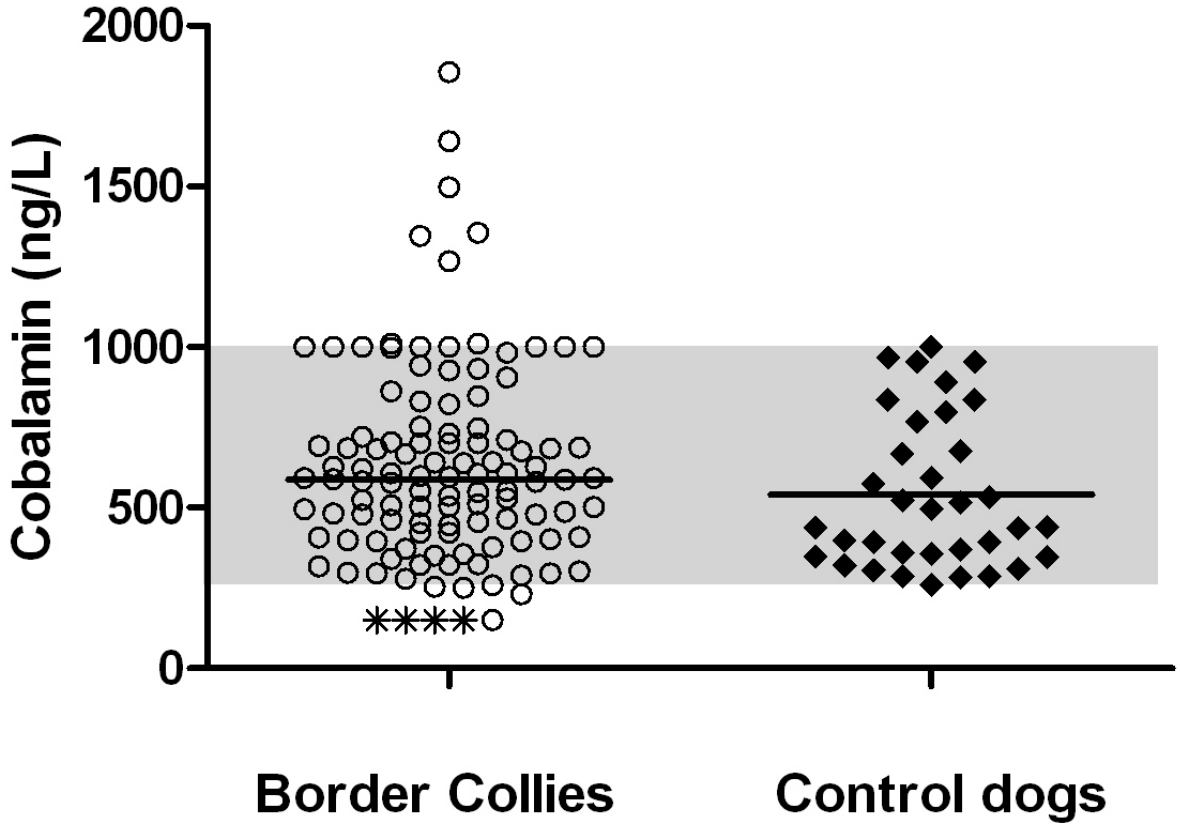

Figure 1–Scatterplot showing results of serum Cbl concentration for BC (n = 113) and control dogs (n = 35). Asterisks indicate the 4 Cbl-deficient BC. Median values are indicated by horizontal lines. The established reference range was 261–1001 ng/L.

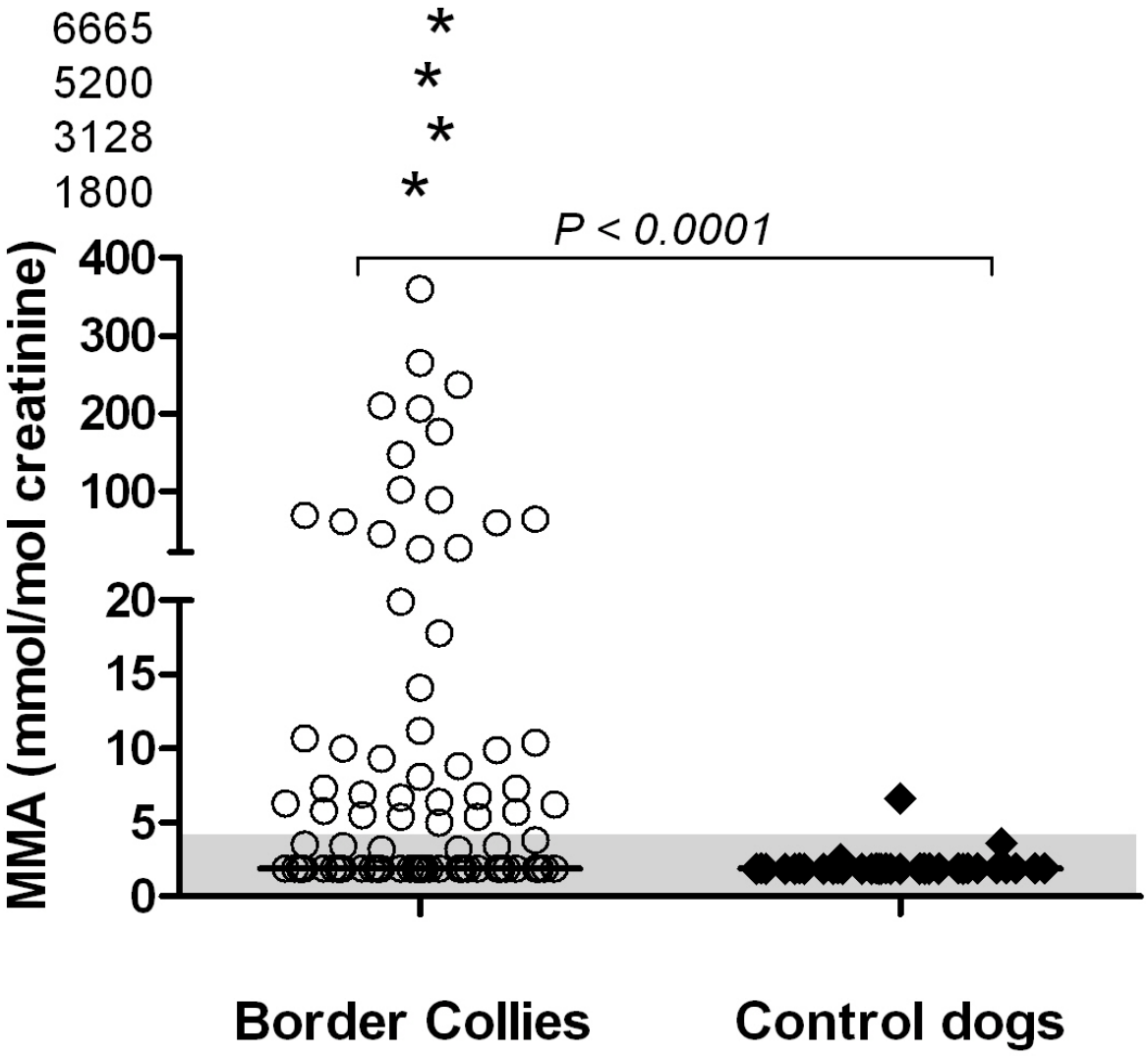

Figure 2–Results of uMMA/Cr of BC (n = 113) and control dogs (n= 35). Asterisks indicate the 4 Cbl-deficient BC. The line indicates the median value. The established upper reference limit was < 4.2 mmol/mol creatinine. uMMA/Cr of 109 healthy BC were significantly higher (*P* < 0.0001)compared to controls.

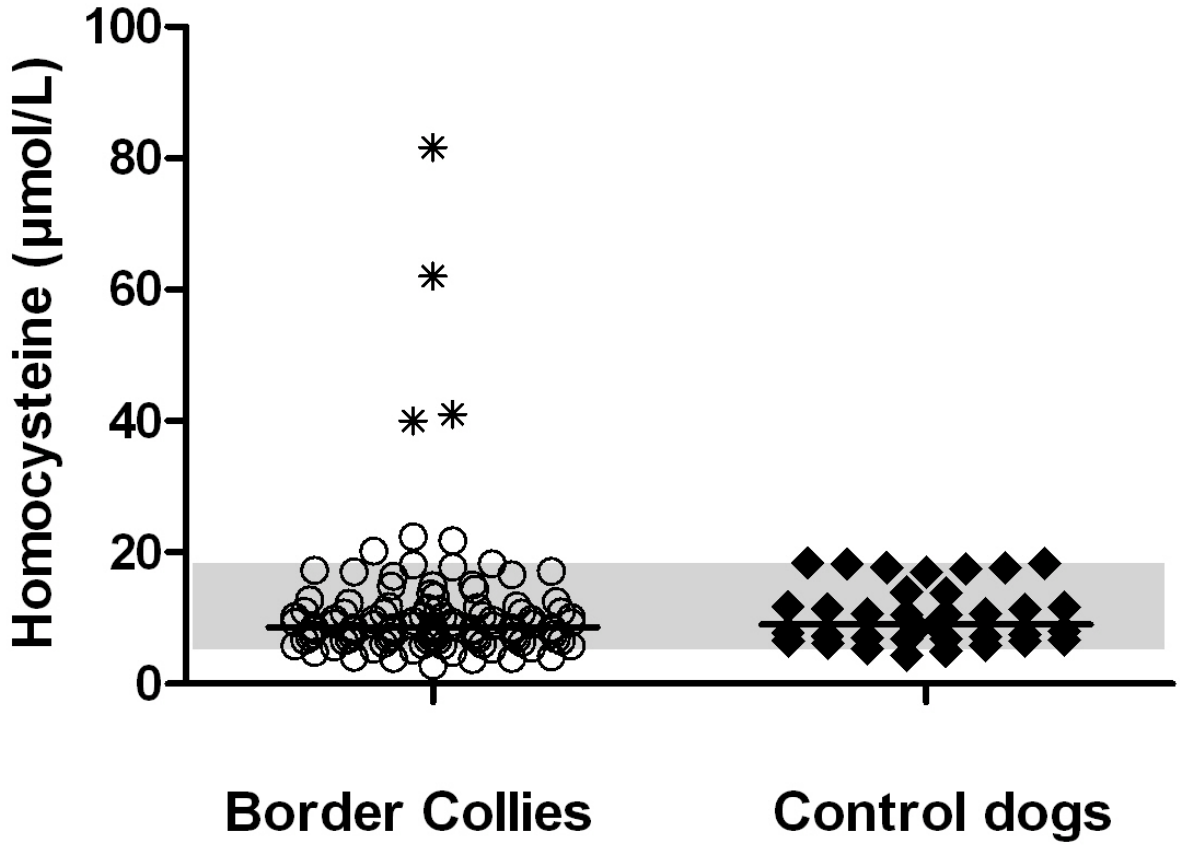

Figure 3–tHcy concentrations of BC (n = 113) and control dogs (n = 35). Asterisks indicate the 4 Cbl-deficient BC. The line indicates the median value. The established reference was 4.3–18.4 µmol/L.

Acknowledgements

An dieser Stelle möchte ich mich bei allen recht herzlich bedanken, die zum Gelingen dieser Arbeit beigetragen haben.

Mein Dank gilt Frau Prof. Dr. Claudia Reusch, die mir diese Dissertation überhaupt ermöglicht hat sowie Herrn Dr. Peter Kook für die hervorragende Betreuung bei der Erstellung der Dissertation.

Ich danke Frau Dr. Sonja Hartnack für ihre Hilfe bei der statistischen Auswertung der Daten.

Bei Herrn Dr. Beat Bigler sowie Herrn Dr. Adrian Sewell bedanke ich mich für die schnelle und zuverlässige Auswertung der Blut- und Urinproben, die einen wesentlichen Teil dieser Dissertation ausgemacht haben.

Ein grosses Dankeschön gilt auch den Mitarbeitern des veterinärmedizinischen Labors in Zürich, die den Mehraufwand, der durch die Auswertung meiner Blutproben entstanden ist, problemlos meisterten.

Bei der Albert-Heim-Stiftung bedanke ich mich für die finanzielle Unterstützung und damit für die Realisierung dieses Projekts.

Vielen Dank an alle Freunde und Bekannte, die mich während dieser Zeit immer vorbehaltlos unterstützt haben sowie an meine Eltern ohne deren Unterstützung

meine Ausbildung und die Erstellung dieser Dissertation nicht möglich gewesen wären.

Ein grosses Dankeschön gilt meinem Freund, Ronny Streubel, der mir in dieser Zeit mit Ratschlägen sowie als geduldiger Zuhörer eine grosse Unterstützung gewesen ist.

Curriculum Vitae

Name	Sabina Lutz
Geburtsdatum	27.03.1985
Geburtsort	Stadt St. Gallen
Nationalität	Schweiz
Heimatort	Wolfhalden AR

1992 – 1998	Primarschule Heimat / Buchwald, Stadt St. Gallen, Schweiz
1998 – 2000	Sekundarschule Blumenau, Stadt St. Gallen, Schweiz
2004	Matura, Kantonsschule am Burggraben, Stadt St. Gallen, Schweiz
2004 – 2009	Studium der Veterinärmedizin, Vetsuisse-Fakultät Universität Zürich, Schweiz
2009	Abschlussprüfung vet. med. Universität Zürich, Schweiz
2009 – 2011	Doktorat an der Klinik für Kleintiermedizin, Vetsuisse-Fakultät, Universität Zürich, Schweiz